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Fats and Function: protein lipid modifications in plant cell signalling

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Abstract

The post-translational lipid modifications N-myristoylation, prenylation and S-acylation are traditionally associated with increasing protein membrane affinity. However this is an over-simplification, with evidence now implicating these modifications in a variety of roles such as membrane microdomain partitioning, protein trafficking, protein complex assembly and polarity maintenance. Evidence for a regulatory role is also emerging, with changes or manipulation of lipid modifications offering a means of directly controlling various aspects of protein function. Proteomics advances have revealed an enrichment of signalling proteins in the lipid-modified proteome, potentially indicating an important role for these modifications in responding to stimuli. This review highlights some of the key themes and possible functions of lipid modification during signalling processes in plants.

Highlights

- Many plant proteins can be N-myristoylated, prenylated, or S-acylated
- Lipid modifications provide more than just a membrane anchor
- Proteomics advances reveal hundreds of proteins to be lipid-modified in plants
- Signalling proteins are enriched in the lipid-modified plant proteome
- Dynamic S-acylation can regulate protein function

Introduction

Membranes define the boundary between a cell and its environment and, within the cell, compartmentalize diverse functions into organelles such as the nucleus, the Golgi and chloroplasts. Membranes are also key in perceiving external signals; integrating information from the environment and translating this into action within the cell. Stimuli such as pathogens, hormones, and environmental conditions are frequently recognised by proteins found at or within membranes, with membrane localisation crucial to their signalling function. While many of these proteins have transmembrane domains to facilitate their localisation, others do not, and may rely on protein-protein interactions, polybasic regions, or post-translational addition of lipids to provide a stable membrane anchor. Lipid modifications can occur on their own, in combination, or in conjunction with transmembrane domains. This latter point hints at a role more complex than that of a simple membrane anchor, with evidence now implicating these modifications in protein-protein interactions, activation state regulation, protein conformation, trafficking and more.

Membranes themselves are now known to be more complex than previously thought, with compelling evidence to suggest that membranes are heterogeneous environments consisting of 'microdomains' with varying physicochemical properties [1,2]. Lipid modifications may promote partitioning of a protein into a specific membrane microdomain environment. Changing the lipidation state of a protein may therefore also alter its specific microdomain affinity, or composition of the microdomain itself, leading to changes in interacting protein availability. This may represent a largely unexplored means of regulating protein complex activity by spatial segregation of component proteins within the membrane. Attempting to understand the interplay between integral membrane proteins, lipid modified proteins and this complex lipid environment is one of the key challenges facing membrane biologists. In this review we will discuss our current understanding, and the future outlook, of how lipid modifications may affect protein function in the intracellular environment.

The major intracellular lipid modifications in plants

Within the cell there are 3 main types of lipid modification whose primary described role is to alter the interaction of a protein with the cytosolic leaflet of membranes; prenylation, N-myristoylation and S-acylation (Figure 1). Prenylation is the addition of either farnesyl or geranylgeranyl chains to defined C-terminal cysteine residue containing motifs, and has recently been reviewed [3]. Unlike in other eukaryotes, mutants in plant prenyl transferase enzymes are viable but show perturbed growth [4]. Prenylation is predicted to affect between 250 and 700 proteins in Arabidopsis [3].

N-myristoylation involves the attachment of the 14-carbon saturated fatty acid myristate to an N-terminal glycine. Myristoylation is catalysed by two N-myristoyl-transferases (NMT1 and 2), with NMT1 being crucial for plant viability while NMT2 is required later for the transition to flowering [5,6]. The Arabidopsis N-myristoylome is predicted to comprise 437 proteins [7].

S-acylation involves the addition of fatty acids, often stearate or palmitate, to cysteine residues through thioester bonds. Proteomics data indicates that S-acylation affects over 500 proteins in Arabidopsis [8] and a similar figure in poplar [9], with recent unpublished data in

Arabidopsis pushing closer to 1000 proteins identified. Interestingly, 50% of S-acylated proteins possess one or more transmembrane domains suggesting that S-acylation acts as more than just a membrane anchor.

A family of 24 S-acyl transferases (PATs) catalyse S-acylation in Arabidopsis. Mutants in PATs show broad developmental phenotypes such as defective cell division and expansion, aberrant root hair growth and early senescence [10-16]. The enzymology of S-acylation in plants has recently been reviewed in detail [17]. Uniquely amongst lipid modifications of proteins, S-acylation has been shown to be reversible [18] although no plant orthologues of mammalian de-S-acylating enzymes have yet been described. Reversibility gives S-acylation the potential to act in a dynamic, regulatory manner during responses to stimuli.

The large numbers of proteins found or predicted to be lipid modified, and the broad phenotypes associated with loss of the lipidation enzymes, highlight the general importance of these modifications in plant cell biology. Lipid-modified proteins are involved in a variety of roles, including cell cycle and growth, cell wall synthesis and intracellular trafficking [19-21]. However, proteomic studies of S-acylation [8,9] and N-myristoylation [22] reveal a marked enrichment in signalling proteins, particularly kinases. This rest of this review will therefore focus on highlighting some examples of the key themes and possible functions of lipid modification during signalling processes in plants.

Lipid modifications in plant cell signalling

Calcium is one of the major secondary messengers in eukaryotic cells, with Calcineurin-B-like proteins (CBLs) acting as key sensors. CBL1 requires N-myristoylation for association with the ER, then subsequent S-acylation to promote trafficking to the PM [23]. Conversely, CBL2 is not N-myristoylated but instead is triply S-acylated to provide tonoplast localisation and a correct response to abscisic acid [24]. Importantly, the N-terminal lipid-modified region of CBL1 or CBL2 alone is sufficient to define their respective PM or tonoplast localisations.

Heterotrimeric G proteins in plants have a broad role in multiple signalling pathways such as development, hormone and light signalling [25]. Mutants in heterotrimeric G protein subunits affect multiple receptor-like kinase (RLK) mediated outputs, such as those mediated by AtFLS2 and AtCERK1, suggesting that heterotrimeric G proteins may act as common signalling factors downstream of RLKs [26]. The γ-subunits AGG1 and 2 are both prenylated and likely S-acylated [27]. The α-subunit AtGPA1 is also dual lipid-modified, with N-myristoyl and S-acyl groups. Lipid modification of AtGPA1 and AtAGG2 subunits is shown to be required for localisation and formation of heterotrimers at the plasma membrane [28]. As RLKs are known to inhabit distinct microdomains [29] it would be interesting to examine whether lipid modifications and, by analogy to mammalian systems, dynamic S-acylation play a role in heterotrimeric G protein signalling. This could be by means of microdomain partitioning, altering interactions with upstream receptors or downstream signalling components.

Thioredoxins (TRX) are involved in redox signalling and stress response. TRX-h9 is both myristoylated and S-acylated, but only N-myristoylation is required for PM localisation [7]. Intriguingly the S-acylated cysteine in the poplar homologue of AtTRX-h9, PtTRX-h4, is required as a reduced thiolate for regenerating the thioredoxin active site in a glutaredoxin

dependant manner [30]. If this is the case then S-acylated AtTRX-h9/PtTRX-h4 will be inactive, as the cysteine residue will not be available to act as the regenerating nucleophile. Even more perplexingly, AtTRX-h9 moves from cell to cell and this requires S-acylation [31]. This raises the possibility that S-acylated and 'inactive' AtTRX-h9 is acting as a cell-to-cell messenger separate to its non-cell-to-cell mobile, non-S-acylated form role as a thioredoxin. The S-acylated mobile form may even be acting as a readout of cellular redox state to neighbouring cells. The most likely route for cell-to-cell movement is through plasmodesmata, which comprise a unique lipid environment particularly enriched in longer chain and saturated fatty acids [32]. S-acyl groups, being long chain saturated fatty acids, may aid in recruitment to, and movement through, the plasmodesmatal membrane environment. This is but one example of the potential, but largely unexplored, interplay between cysteine modifications and redox sensing.

Lipid modifications in plant immune signalling

Immune signalling in plants is mediated by extracellular receptors (frequently RLKs) or intracellular proteins (frequently NB-LRRs, often termed R-proteins). These recognise conserved features of the pathogen, such as bacterial flagellin, or pathogen-secreted virulence factors known as 'effectors'. Proteomic studies of S-acylation and N-myristoylation have revealed numerous examples of defence-related proteins being modified [8,9,22], indicating a prominent role for lipid modifications in immune signalling. Reduced N-myristoylation in a NMT1 low-expressing mutant compromises the immune response [33], likely due to a decrease in the PM-enrichment of several defence-associated proteins [34].

The best-characterised plant immune receptor, the LRR-RLK FLS2, is S-acylated. FLS2 is an integral membrane protein and S-acylation does not affect subcellular localisation, suggesting an alternative but as yet unknown role for S-acylation [35]. The FLS2-associated receptor-like cytoplasmic kinase (RLCK), BIK1, requires N-myristoylation for its PM localisation and function in immune signalling [36]. Notably, N-myristoylation mutants of BIK1 exhibit higher auto-phosphorylation activity, potentially as a result of loss of interaction with its integral membrane negative regulator AtPP2C38 [37]. BIK1 is also S-acylated, and while no role has been ascribed to S-acylation here it is tempting to speculate that it may aid in formation of a complex with S-acylated FLS2.

Lipid modifications affecting multiple proteins in a pathogen recognition event appears to be a common theme. The NB-LRR protein RPS5 triggers cell death upon detecting PBS1 cleavage by the *Pseudomonas syringae* effector AvrPphB. PBS1 [38] and RPS5 [39] are N-myristoylated and S-acylated to recruit them to the PM, while AvrPphB and family members are cleaved *in planta* to reveal S-acylation and N-myristoylation sites to allow PM localisation [40]. AvrPphB mutants lacking these sites lose their virulence and are not recognised by the plant, as disrupting membrane localisation of AvrPphB prevents PBS1 cleavage and RPS5 activation. This is an example of lipidation becoming part of the plant-pathogen arms race, where each uses lipidation of effector or guard proteins to target a lipidated plant susceptibility factor.

Whilst immune signalling is a crucial aspect of plant survival, it must be carefully controlled to prevent unnecessary activation. The rice RLCK OsPTI1a is a PM-localised negative regulator

of immunity in plants [41]. Mutation of putative S-acylated cysteines at the N-terminus abolished membrane localisation and altered the proteins found to be complexed with PTI1a, with several immunity-related proteins found to be lacking [42]. This suggests that S-acylation is required in this case for establishing or increasing the efficiency of protein-protein interactions, likely by targeting and concentrating each component into the same membrane or microdomain.

Dynamic lipid modifications in plant signalling?

The best-characterised dynamic lipid modification in plants is that of the Rho-related GTPase ROP6. ROPs are implicated in a myriad of signalling processes, including developmental, biotic/abiotic stress, hormone and calcium signalling. ROP6 is prenylated, which determines PM localisation, but is also S-acylated in an activation-state dependant manner, with S-acylation confined to the active population of ROP6. Expression of constitutively active ROP6 results in short, swollen root hairs with a loss of tip-focussed reactive oxygen generation. Mutation of the S-acylation sites in ROP6 supresses the effects of the constitutively active mutation [43], indicating that S-acylation is required for active ROP function. S-acylation alters the physical properties of ROP6, as judged by partitioning into Triton X-100 detergent-resistant membrane fractions [44]. It is therefore possible that S-acylation of ROP6 either allows access to, or creates, a membrane microdomain containing downstream signalling factors. Notably, ROP proteins also contain a polybasic domain in the vicinity of lipid-modified residues, which in ROP10 has been shown to be required for PM localisation [45]. These domains may be required for subsequent lipid modification of the protein, and could potentially play a role in microdomain localisation; polybasic regions in mammalian GTPases have been shown to associate with specific phosphatidyl phosphoinositides in the plasma membrane [46].

The RLCK SGN1 is required in roots for formation of a functional Casparian strip. SGN1 is found exclusively on the cortical face of the endodermal plasma membrane. S-acylation at the N-terminus is required for localisation, with mutants lacking S-acylation sites rendered cytoplasmic and unable to complement the *sgn1* mutant. SGN1 appears to undergo cycles of de-S-acylation and S-acylation [47]. This likely defines its strictly polar localisation; S-acylation acts to trap SGN1 at the desired PM surface, while de-S-acylation and removal from the PM at the limits of SGN1s desired distribution spatially restricts signalling. In this manner S-acylation can be used to set up asymmetric distributions of signalling molecules, a process important for determining cell polarity.

Whilst N-myristoylation and prenylation are not enzymatically reversible, there remains the possibility of a regulatory role, although confirmed examples in plants are few. Binding of the lipid moiety, either by the protein itself or by an interacting protein, has the potential to alter protein activity. For example, RhoGDI proteins bind the prenyl group of inactive ROPs to sequester them in the cytoplasm or help define polar ROP distributions at the membrane [48]. ARFs, small GTPases regulators of vesicle biogenesis, undergo activation dependant conformational change that exposes or shields an N-myristoyl group to alter membrane association [49]. As plant ARFs are N-myristoylated and show cycling between the cytosol and

golgi membrane, it is reasonable to suggest that a myristoyl switch may well be operating [34].

Future outlook

The data and ideas outlined above demonstrate that the traditional theory - that lipid modifications of proteins merely act as membrane glue - is now outmoded. These modifications, while relatively simple additions, are potentially multi-functional and have huge scope for regulating protein function in numerous ways; whether through localisation to membranes or specific microdomains within them, affecting protein activity, complex formation and protein-protein interactions, maintaining protein conformation or stability. The variety of known roles for lipid modifications in plant signalling proteins are represented in **Figure 2.** Even the presumption that lipid modification automatically leads to membrane association is a risky one to make. Several proteins reportedly do not associate with membranes after lipid modification. The N-myristoylated tomato kinase Pto provides resistance against AvrPto expressing *Pseudomonas syringae*. Interestingly N-myristoylation does not promote Pto membrane association but appears to act as a switch, either inhibiting kinase activity in the absence of pathogen or promoting interaction with downstream proteins after AvrPto binding [50]. Both the transport particle BET3 and the mammalian transcription factor TEAD family use S-acyl groups as hydrophobic scaffolds and S-acylation is essential for correct folding of these proteins [51,52]. It remains to be seen how many other lipidated proteins have what are normally considered atypical uses for lipid groups in their function.

The potential for lipid modifications to alter protein function in response to a stimulus, in an analogous way to phosphorylation or ubiquitination, is a particularly exciting avenue of research. This is really only just beginning to be opened up and many questions remain unanswered. One particular area in need of investigation is how lipid modifications interact with membrane microdomains and whether lipid modification of a protein can alter the microdomain environment around it (reviewed [2]). This may be especially relevant to large protein complexes with multiple lipid modifications, such as S-acylation of the cellulose synthase complex, hypothesised to contain close to 100 S-acyl groups attached to an 18mer complex [19]. Changes to the lipid environment surrounding a protein or complex, caused by a change in lipidation state, could result in other proteins being recruited or excluded from the resulting proteolipid complex based purely on the physical properties of the proteins in question. In silico simulations show that S-acylation can alter the ‘tilt’ of a transmembrane domain relative to the lipid bilayer [53]. This could potentially affect protein function by exposing or hiding an interaction site, blocking the pore of a channel, etc. The tilting of a helix may also influence specific microdomain formation or occupancy, by restricting which lipid species are able to pack around the helix (reviewed [54]).

As well as the potential wealth of protein regulation that may occur through N-myristoylation, S-acylation and prenylation, there is also the possibility of as yet unidentified lipid modifications in plants. N-palmitoylation, O-palmitoylation and octanoylation can be catalysed by members of the MBOAT family, and modification by cholesterol addition has also been shown to exist in mammalian systems (reviewed [55]) but have not yet been

investigated in plants. In addition, curiosities such as Histone H3 S-acylation at the eukaryotically conserved cys110 residue bear further investigation; there is no known S-acylating enzyme in the nucleus, and the potential of a role for dynamic S-acylation in chromatin organisation is particularly exciting.

We now often have the luxury of access to information on every gene and predicted protein in our species of interest. Post-translational modifications add the next layer of complexity, creating the opportunity to expand the function of the proteome by offering diverse means of regulation. Among the thousands of proteins now shown or predicted to be lipid-modified in plants, relatively few examples yet boast a complete story to explain why.

Recent technological advances, such as super-resolution microscopy and single-molecule imaging, will undoubtedly enhance our understanding of lipid modified proteins and membrane microdomains in the future. Combining these approaches with the increasing predictive power of databases, breakthroughs in the structural biology of membrane proteins and the emerging data on regulatory aspects of lipid modifications ensures that the field is set for fascinating developments.

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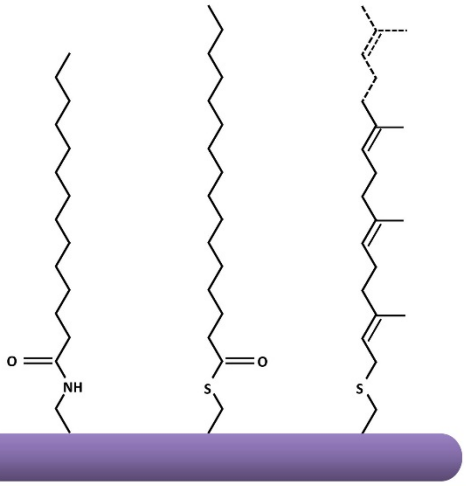
FIGURE LEGENDS



	N-myristoylation	S-acylation	Prenylation
Lipid group	myristate	palmitate, stearate	farnesyl, geranylgeranyl
Linkage	amide	thioester	thioether
Amino acid	Glycine (N-term)	Cysteine (any)	Cysteine (near C-term)
Enzymes	NMT1, NMT2	PATs (x24 in Arabidopsis)	PFT, PGGT1, PGGT2
Membrane affinity	low	high	Farnesylation med geranylgeranyl high
Reversible?	NO	YES	NO
Consensus sequence	Loosest consensus (M)GXXXS/TXX	none	C-terminal CaaX (PFT/PGGT1) CCXX, XCXC, XCCX or XXCC (PGGT2)

Figure 1: Overview of intracellular lipid modifications in plants

For S-acylation, 18 carbon stearate is shown. For prenylation, a farnesyl group is shown (solid lines), with an additional isoprene unit (dashed lines) to depict the geranylgeranyl group. For consensus sequences, (M) indicates the Methionine initiator, 'X' indicates any amino acid, and 'a' indicates any aliphatic amino acid.

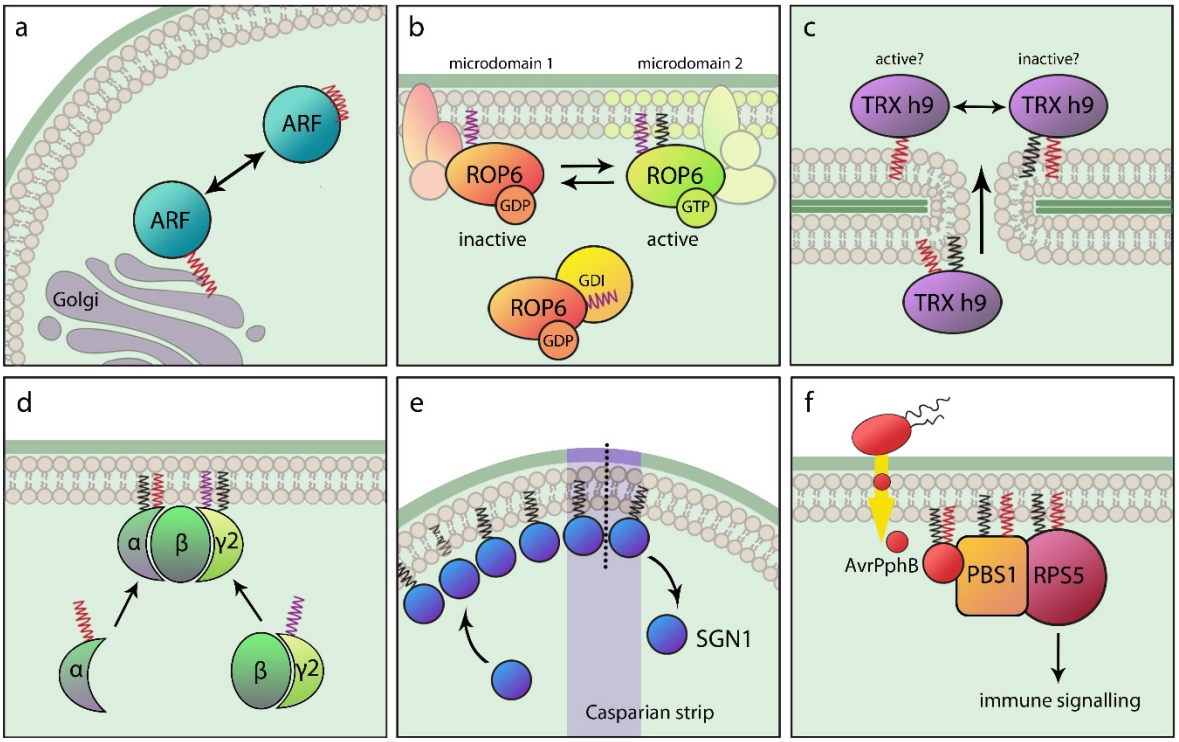


Figure 2: Examples of lipid modified proteins in plant cell signalling

The lipid modifications N-myristoylation (red zig-zag), Prenylation (purple zig-zag) and S-acylation (black zig-zag) have been shown to affect protein function in a variety of ways. (a) ARF GTPases cycle between the Golgi and the cytoplasm, potentially by means of a 'myristoyl switch', where changes in protein conformation lead to the exposure or sequestration of the myristoyl group. (b) ROP6 is prenylated, and undergoes dynamic S-acylation that correlates with activation state. Active, S-acylated ROP6 is hypothesised to occupy a different membrane microdomain environment to inactive, non-S-acylated ROP6. Inactive ROP6 can also be sequestered into the cytosol by RhoGDI proteins binding the prenyl group. (c) TRX-h9 requires N-myristoylation for plasma membrane (PM) localisation and additionally requires S-acylation for cell-cell movement. S-acylation is also proposed to inhibit thioredoxin activity suggesting a regulatory interplay between S-acylation mediated movement and cellular redox state. (d) A heterotrimeric G-protein is comprised of α, β and γ subunits, which are differentially lipid modified and contribute to heterotrimer formation at the PM. (e) SGN1 localises specifically to the cortical face of the endodermal plasma membrane, where it undergoes cycles of S-acylation and de-S-acylation, potentially to maintain polarity and determine location of the casparian strip in roots. (f) The *Pseudomonas syringae* effector AvrPphB is secreted into the plant, cleaving PBS1 and triggering immunity through the R protein RPS5. All three proteins require both N-myristoylation and S-acylation for PM colocalisation.

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